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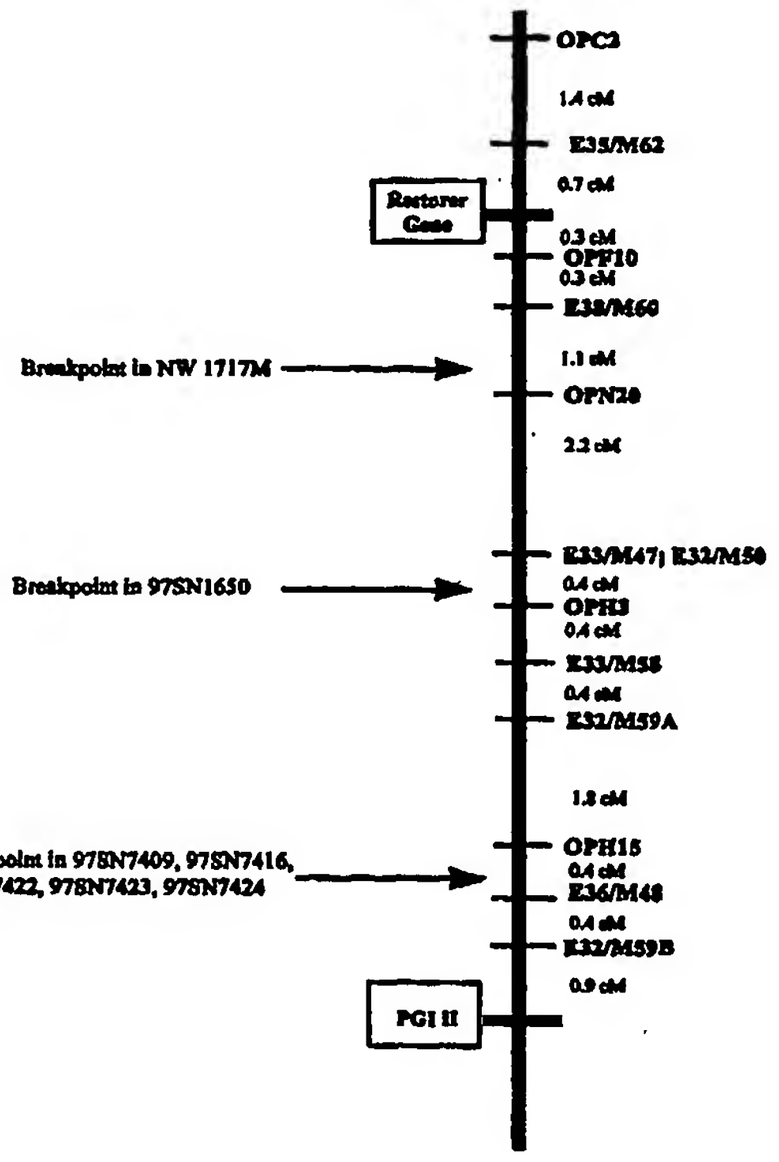
(54) Title: USE OF MOLECULAR MARKERS FOR GENOTYPE DETERMINATION OF THE OGURA Rf GENE IN BRASSICA NAPUS

(57) Abstract

This invention relates to *Brassica* plants and methods of breeding *Brassica* plants. More particularly, the invention relates to the use of molecular markers for determining the genotype of the Ogura Rf gene in *Brassica* plants at an early stage in their development. The method involves using a combination of primers derived from RAPD markers in PCR reactions to allow discrimination between homozygous, heterozygous and non-restorer or sterile genotypes. Also disclosed are combinations of primers which allow such discrimination.



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USE OF MOLECULAR MARKERS FOR GENOTYPE DETERMINATION OF THE OGURA Rf GENE IN BRASSICA NAPUS

Field of the Invention

This invention relates to *Brassica* and *Brassica* breeding. More particularly, the invention relates to *Brassica* lines having the Ogura restorer (Rf) gene, and methods to identify these lines using genetic marker analysis.

Background of the Invention

Brassica plants, and in particular oilseed from such plants, are increasingly important crops. As a source of vegetable oil, they presently rank behind only soybeans and palm in commercial importance and they are comparable with sunflowers. The oil is used both as a salad oil and as a cooking oil.

Significant improvement in yield has been demonstrated in *Brassica* using single cross hybrids (Hutcheson et al. 1981, Sernyk and Stefansson 1983; Grant and Beversdorf 1985). For commercial hybrid production, a reliable and efficient pollination control system is required. In *Brassica*, a number of pollination control systems are available: nuclear male sterility, self incompatibility and cytoplasmic male sterility (CMS). There are different types of CMS in *Brassica*, including, for example, Ogura CMS and Polima CMS.

CMS in plants is characterized by failure to produce normal anthers and functional pollen. It is a maternally inherited trait. The genetic determinants for CMS reside in the mitochondrial genome as opposed to nuclear genome. A 2.5 Kb NCO1 fragment that gives rise to a 1.4 Kb transcript, has been implicated in Ogura CMS (Bonhomme et al. 1991). This transcript contains two open reading frames, ORF 158 and ORF 138. Subsequent study seems to have eliminated the role of ORF 158 and hence reaffirmed the key role of ORF 138 in pollen abortion in Ogura CMS plants (Bonhomme et al. 1992).

To be useful for F_1 hybrid seed production, a CMS system requires strong fertility restorer and sterility maintainer alleles. What distinguishes the Ogura system from other CMS systems in *Brassica* in terms of use for commercial hybrid production is the availability of a workable restorer or Rf gene (in this application, "OGU Rf").

Both Ogura CMS and a good source of fertility restorer gene have been identified in radish *Raphanus sativus*, (Ogura 1968; Heyn 1976). Ogura CMS has been transferred from radish to *B. napus* by inter-specific crossing, embryo rescue and backcrossing (Bannerot et al. 1974). Protoplast fusion with *B. napus* was required to produce male sterile *B. napus* cybrids (Pelletier et al. 1983). The restorer gene has also been transferred from radish to *B. napus* by intergeneric crossing (Heyn 1976), and fully restored Ogura CMS plants were identified carrying a single restorer gene (Pelletier et al. 1987). Unfortunately, decreased female fertility in the restored plant accompanied such a transfer and it was postulated that the radish chromosome segment that carried the restorer gene and that was introgressed from radish also contained radish DNA with genetic information other than the Ogura restorer gene (Pellan-Delourme and Renard 1988). Although retention of radish genetic information around the restorer allele has been recently reduced so as to improve seed production, (Delourme et al. 1991), a linkage study by Delourme and Eber 1992 identified an isozyme locus (marker), PGI II, linked to the restorer gene and presumably also located on the radish chromosome segment. A biochemical assay is required to determine PGI II expression. PGI II is expressed as a protein and is analyzed by running out the protein on a gel. By analyzing the patterns on the gel it is possible to determine the PGI II genotype of individual samples.

Genetic marker analysis has proven useful for detecting the presence of the restorer gene. Without markers, such as PGI II or genetic markers, breeders need to wait until the plants have flowered to determine whether they carry the restorer gene or not. If the restorer gene is present, the flowers are normal (fertile) and if the restorer gene is absent, they have reduced (abnormal/nonfunctional) anthers and lack pollen.

There are significant advantages to identifying the presence of a gene by marker analysis in a segregating population at an early stage in a plant's development. One advantage is that by determining a plant's genotype before it flowers, the breeder saves resources (time and space) associated with growing the plant to flowering and eliminating undesired genotypes. In winter *napus*, the ability to identify the presence of a gene by marker analysis enables the breeder to identify the plants in which the gene is present before vernalization. This is particularly helpful since it allows breeders to eliminate any plants, prior to vernalization, which do not carry the gene, saving valuable space in vernalization

rooms. Winter *B. napus* requires an eight to twelve week period of vernalization in order to initiate flowering.

Markers can be used not only to determine the presence of a gene but also its genotype (homozygous vs. heterozygous). Ideally, if the sequence of the actual gene is available, one can use molecular techniques to definitively determine its presence by PCR analysis. Obtaining a gene sequence is difficult, but identifying loci (markers) that are linked to the gene, though difficult, is less difficult compared to identifying the gene sequence. The "mapping distance" of marker loci to the gene of interest (termed "linkage") will determine the level of confidence in concluding that the gene is present when the marker has been identified as being present. The shorter the "mapping distance" the greater the level of confidence.

The isozyme locus identified by Delourme and Eber (1992) can be used to identify the presence of the restorer gene and this isozyme analysis is often used to determine the genotype of plants containing the OGU Rf gene. However, using this type of marker has certain limitations. The analysis involves assaying a gene product, a protein, which is easily degraded if leaf tissue samples or extracts prepared from leaf tissues are not handled under ideal temperature conditions. In addition, the gene product may be developmentally regulated, i.e. not expressed in certain tissues or at a certain developmental stage. Other PGI loci (for example, the PGI II isozyme locus) are present in *B. napus* and, depending on the combination of alleles present in a particular plant, the banding pattern of the PGI-II protein product run out on the gel becomes complex. as has been found in spring *napus*. Thus, it may not be possible to associate enzyme phenotype with genotype of the restorer gene. Isozyme analysis has been used extensively in winter *napus* populations, but does not give reliable results in spring *napus* populations due to the complexity of the banding patterns observed on the gels.

At present, there is no technology or method, other than isozyme analysis, which uses molecular/biochemical markers to determine the genotype of the OGU Rf gene in *Brassica*. PCT International patent application WO 97/02737 of Forman, et al., published on January 30, 1997, claims a method of producing an improved restorer line of Brassica wherein testing the progeny for fertility included the use of AFLP, RFLP and/or RAPD molecular markers, microsatellites, primer and other probes to give genetic fingerprints

of *Raphanus sativus* material. In one embodiment, the molecular markers are mapping to a similar region as that of OPC2. Although the molecular markers disclosed indicate the presence of the restorer gene, they do not indicate the absence of the restorer gene, nor determine the genotype of the restorer gene, as do the molecular markers of the present invention.

In the Delourme et al. (1994) study, six random amplified polymorphic DNA (RAPD) markers were used in a bulk segregant analysis. However, unlike the present invention of Sequenced Characterized Amplified Region (SCAR) markers, (i) the RAPD markers were generated by the amplification of genomic DNA using only a single primer of arbitrary nucleotide sequence to drive the amplification reaction and, (ii) only the presence of the restorer gene (Rf) in plants containing the Ogura CMS system of rapeseed could be determined. The Delourme et al. (1994) study did not teach one skilled in the art how to determine the specific genotype of the variety.

The Delourme et al (1994) study mentioned the possibility of SCAR markers from the Randomly Amplified Polymorphic DNA (RAPD) fragments which were found to be linked to the restorer locus and postulated that these markers could then be used to facilitate breeding programs involving this gene. Delourme et al. (1994) did not teach one skilled in the art (i) how to use markers to determine the genotype of *Brassica* plants for Ogura CMS, (ii) how to develop SCAR markers from the 'rf' associated RAPD markers, (iii) how to develop and use a combination of SCAR markers in a single, multiplex amplification reaction to determine the specific genotype of a plant variety (rather than simply determining whether a plant variety has a fertility restorer locus or a fertility maintainer locus), or (iv) how to develop and use SCAR markers linked to the absence of the restorer locus, where the markers are derived from OPY5. Without SCAR markers from the 'rf' associated RAPD markers, one would be unable to differentiate reliably between a plant that is homozygous or heterozygous for the Rf gene.

The weaknesses of current technology and methods for determining the genotype of the OGU Rf restorer gene in *Brassica* are that:

i) isozyme analysis is ineffective, especially in spring *Brassica napus*, for determining OGU Rf genotype because the analysis involves assaying a protein which may degrade or which may be developmentally regulated. Depending on the combination of alleles

present in a particular plant, the banding pattern of the protein product may be too complex to interpret (as is the case for spring *Brassica* napus).

ii) RAPD markers are unreliable since reactions are performed at low annealing temperatures. Slight variations in reaction conditions or components, for example, DNA quality, or concentration of primer or DNA, often results in total or partial failure of RAPD products to be amplified.

iii) RFLP markers are cumbersome in breeding programs because a large amount of DNA is required, a large number of samples is required for throughput, and a slow turn-around time is associated with RFLP analysis.

iv) utilizing SCAR markers linked to the restorer gene (Rf) allows one to distinguish varieties carrying the restorer gene from varieties not carrying the restorer gene. The markers will show up in individuals carrying the restorer gene but will be absent in individuals not carrying the restorer gene. However, this system:

- does not differentiate between homozygous 'RfRf' and heterozygous 'Rfrf' restorer types. The marker will show up in both of these genotypes since they both contain the restorer gene; and

- can provide false negatives. An individual in which the marker does not show up would score as 'rfrf' indicating that it does not carry the restorer gene. In fact, the PCR reaction may have simply failed (for any one of a number of reasons) - this is a fairly common phenomenon in PCR based systems.

It is an object of the present invention to provide a simple, efficient and reliable method for identifying the genotype of *Brassica* germplasm for the Ogura restorer gene.

It is another object of the present invention to identify markers that can be used in combination to determine the genotypic state of the gene in segregating populations, including both winter and spring *Brassica napus* populations, as well as in other *Brassica* species.

It is another object of the present invention to distinguish between homozygous and heterozygous restorer types by using a combination of standard PCR primers to amplify markers for the restorer gene 'Rf' and for the "absence of restorer gene" 'rf'.

It is another object to provide a method to rapidly screen large numbers of samples of *Brassica* germplasm to determine the genotype for the OGU restorer gene, without having to grow the plant to maturity (i.e. before the plant flowers).

It is a further object to characterize low glucosinolate recombinant Ogura Rf lines by identifying breakpoints and hence determining the amount of *Raphanus* DNA retained by those recombinant lines.

Summary of the Invention

This invention relates to a method for determining the genotype of *Brassica* germplasm for the Ogura (Rf) restorer gene in a *Brassica* breeding program, comprising the steps of: (1) amplifying the *Brassica* germplasm for the Ogura (rf) restorer gene using at least one primer; and (2) determining the genotype using at least two nucleic acid markers, wherein one marker indicates the presence of the OGU Rf gene (Rf) and the other marker indicates the absence of the OGU Rf gene (rf), and using a dot blot assay to detect the genotype. The *Brassica* germplasm may be a winter or spring *Brassica napus, Brassica rapa* or *Brassica juncea.*

The markers may be SCAR markers, RAPD markers or AFLP markers. The SCAR marker indicating the absence of the OGU Rf gene (rf) may be Y5, a marker having partial homology to the Y5 sequence, or any other marker selected from a sequence of the RAPD band from which that marker is derived. The SCAR marker indicating the presence of the OGU Rf gene (Rf) may be C2, N20, F10, a marker having partial homology to one of these sequences, or any other marker selected from a sequence of the RAPD band from which these markers are derived. The RAPD marker indicating the absence of the OGU Rf gene (rf) may be OPY5, OPG8, OPG2, a marker having partial homology to one of these sequences, or any other marker selected from a sequence of the band from which these markers are derived. The RAPD marker indicating the presence of the OGU Rf gene (Rf) may be OPC2, OPN20, OPH3, OPF10, OPH15, a marker having partial homology to one of these sequences, or any other marker selected from a sequence of the band from which these markers are derived. The AFLP marker indicating the presence of the OGU Rf gene (Rf) may be E36XM48AIII, E35XM62AV, E33XM47AI,

E38XM60AI, E32XM50, E32XM59A, E32XM59B, E33XM58, a marker having partial homology to one of these sequences, or any other marker selected from a sequence of the band from which these markers are derived.

The amplification step in the method to which this invention relates may comprise at least one polymerase chain reaction (PCR) and primers for PCR, which may be primers described in Table 2 below or primers having partial homology to those primers. The method involving this amplification step may also comprise a multiplex polymerase chain reaction (PCR) using all of the primers in a single reaction.

The amplification step may also comprise using primer sets C2 and Y5 in combination in a multiplex polymerase chain reaction (PCR), and the step of determining the genotype may comprise:

(A) the steps of (1) dotting PCR reaction onto two identical membranes, (2) probing the membranes with probes, wherein one membrane is probed with a probe which hybridizes to a marker indicating the presence of Rf (which may be a marker of 677 base pairs) and the second membrane is probed with a probe which hybridizes to a marker indicating the absence of rf (which may be a marker of about 774 base pairs), and (3) comparing the two membranes to determine the genotype of *Brassica* germplasm for the Ogura restorer; or

(B) the steps of (1) running out the products of PCR on an electrophoresis gel, wherein the reaction products may be (a) one band (about 677 bp), indicating a genotype of RfRf, (b) two bands (about 677 bp and about 774 bp), indicating a genotype of Rfrf, or (c) one band (about 774 bp), indicating a genotype of rfrf, and (3) reading the gel to determine the genotype of *Brassica* germplasm for the Ogura restorer.

The invention also includes a homozygous locus associated with the presence of the Ogura (RfRf) restorer gene (said locus mapping to at least one of the markers C2, N20, F10, a marker having partial homology to one of these sequences, or any other marker selected from a sequence of the RAPD band from which these markers are derived) or a homozygous locus associated with the absence of the Ogura (rfrf) restorer gene (said locus mapping to one of the markers Y5, a marker having partial homology to that sequence or any other marker selected from a sequence of the RAPD band from which that marker is derived). A heterozygous locus associated with the presence of the Ogura

(Rfrf) restorer gene and that maps to one of the aforementioned markers is also part of this invention.

The invention also includes a homozygous locus associated with the presence of the Ogura (RfRf) restorer gene (said locus mapping to at least one of the markers OPC2, OPN20, OPH3, OPF10, OPH15, E36XM48AIII, E35XM62AV, E33XM47AI, E38XM60AI, E32XM50, E32XM59A, E32XM59B, E33XM58, a marker having partial homology to one of these sequences, or any other marker selected from a sequence of the RAPD or AFLP band from which these markers are derived) or a homozygous locus associated with the absence of the Ogura (rfrf) restorer gene (said locus mapping to one of the markers OPY5, OPG8, OPG2, a marker having partial homology to that sequence or any other marker selected from a sequence of the RAPD or AFLP band from which these markers are derived). A heterozygous locus associated with the presence of the Ogura (Rfrf) restorer gene and that maps to one of the aforementioned markers is also part of this invention.

A combination of markers for determining the genotype of *Brassica* germplasm for the Ogura (Rf) restorer gene is part of this invention, said combination comprising at least one marker that indicates the presence of the Ogura (Rf) restorer gene and at least one marker that indicates the absence of the Ogura (rf) restorer gene. The combination of markers may also comprise a first set of nucleic acid markers, which may be C2, N20, F10, a marker having partial homology to one of these sequences, or any other marker selected from a sequence of the RAPD band from which these markers are derived, and a second set of nucleic acid markers, which may be Y5, a marker having partial homology to the Y5 sequences, or any other marker selected from a sequence of the RAPD band from which that marker is derived. The combination of markers may further comprise a first set of nucleic acid markers, which may be OPC2, OPN20, OPH3, OPF10, OPH15, E36XM48AIII, E35XM62AV, E33XM47AI, E38XM60AI, E32XM50, E32XM59A, E32XM59B, E33XM58, a marker having partial homology to one of these sequences, or any other marker selected from a sequence of the RAPD or AFLP band from which these markers are derived), and a second set of nucleic acid markers, which may be OPY5, OPG8, OPG2, a marker having partial homology to one of these sequences, or any other marker selected from a sequence of the RAPD or AFLP band from which these markers are derived).

The invention also relates to SCAR markers: (1) for determining the presence of the Ogura (Rf) restorer gene in the genotype of *Brassica* germplasm, which may be the nucleic acid marker C2, N20, F10, a marker having partial homology to one of these sequences, or any other marker selected from a sequence of the RAPD band from which these markers are derived; or (2) for determining the absence of the Ogura (rf) restorer gene in the genotype of *Brassica* germplasm, which may be the nucleic acid marker Y5, a marker having partial homology to that sequences, or any other marker selected from a sequence of the RAPD band from which that marker is derived.

A RAPD marker for determining the presence of the Ogura (Rf) restorer gene in *Brassica* germplasm, which may be the nucleic acid marker OPN20, OPH3, OPF10, OPH15, a marker having partial homology to one of these sequences, or any other marker selected from a sequence of the band from which these markers are derived, is also part of this invention.

This invention also relates to AFLP markers for determining the presence of the Ogura (Rf) restorer gene in *Brassica* germplasm, which may include the nucleic acid marker E36XM48AIII, E35XM62AV, E33XM47AI, E38XM60AI, E32XM50, E32XM59A, E32XM59B, or E33XM58 a marker having partial homology to one of these sequences, or any other marker selected from a sequence of the band from which these markers are derived.

The invention also includes a combination of primers for use in a polymerase chain reaction (PCR) to determine the genotype of *Brassica* germplasm for the Ogura (Rf) restorer gene, which primers may be those described in Table 2 below, primers having at least partial homology to those primers, primers which anneal to a sequence from which those primers are derived, or primers derived from any portion of the RAPD fragment or amplified RAPD fragment from which those primers are derived. A primer kit comprising the aforementioned primers is also part of this invention.

In addition, this invention includes a system for determining the genotype of *Brassica* germplasm for the Ogura (Rf) restorer gene, comprising the steps of:

(a) amplifying the genotype in a single multiplex polymerase chain reaction (PCR), using primers described in Table 2 below, primers having at least partial homology to those primers, primers which anneal to a sequence from which those primers are derived, or primers derived from any portion of the RAPD fragment or amplified RAPD fragment from which those primers are derived; and

(b) determining the genotype using a combination of nucleic acid markers, wherein the markers may be a C2 marker indicating the presence of the OGU Rf gene (Rf), or markers having at least partial homology with a C2 marker, and a Y5 marker indicating the absence of the OGU Rf gene (rf), or markers having at least partial homology with a Y5 marker.

Brief Description of the Drawings

In drawings which illustrate embodiments of this invention,

FIG. 1 illustrates by way of exemplification RAPD markers linked to the Ogura restorer (Rf) gene by use of the original F2 population 94CWN2133.

FIG. 2 illustrates by way of exemplification RAPD and AFLP markers linked to the Ogura restorer (Rf) gene by use of the second section of population 94CNW2133.

FIG. 3 illustrates by way of exemplification a map of RAPD and AFLP markers associated with the presence of the Ogura Restorer gene, indicating the breakpoint positions in several recombinant *Brassica napus* lines.

These and other objects and advantages of the invention will be apparent to those skilled in the art from the following description and appended claims.

Detailed Description of the Invention

This invention enables a breeder to distinguish between homozygous and heterozygous Ogura restorer types by using a combination of two SCAR markers, one linked to the restorer gene 'Rf' and one linked to 'rf', i.e., absence of restorer gene.

In one embodiment, two PCR reactions for each DNA sample are used, one with primers for the 'Rf' marker and one with primers for the 'rf' marker. By comparing the results of these two reactions it is possible to determine the genotype of individual plants, either 'RfRf', 'Rfrf', or 'rfrf'. Homozygous restorer types 'RfRf' would contain only the marker linked to 'Rf'. Heterozygous plants would contain the markers linked to both 'Rf' and 'rf'. Homozygous non-restorer types would contain only the marker linked to 'rf'.

In another embodiment, a single PCR reaction for each DNA sample is used, by multiplexing both sets of primers in the single PCR reaction. The two sets of primers, when used together in a multiplex PCR reaction, allow for genotypic determination of the OGU Rf gene in individual plants of segregating OGU Rf populations.

These two sets of standard PCR primers were derived via bulked segregant analysis of a segregating OGU Rf population using RAPD PCR (Example 1) and AFLP analysis (Example 7). RAPD and AFLP markers (bands) linked to the OGU Rf gene (Rf) were discovered. Other RAPD and AFLP markers were found to be present only in the absence of OGU Rf (rf). The markers (bands) of interest were cloned and sequenced and the standard (SCAR) PCR primers were designed from these sequenced fragments.

One standard PCR primer pair, derived from a RAPD band generated using the Operon primer OPC2, gives a band which is linked to the OGU Rf gene (Rf). The second primer pair, derived from a RAPD band generated using the Operon primer OPY5, gives a band which is only present in the absence of the restorer gene, i.e., is linked to rf. The two sets of primer pairs designated as C2 and Y5, derived from RAPD markers using OPC2-linked to Rf and using OPY5-linked to rf are described below in Table 2.

When used in standard PCR reactions, primer pair C2 gives a band of 677 bp (base pairs) indicating the presence of the OGU Rf gene (Rf). Primer pair Y5 gives a band of 774 bp indicating the absence of the OGU Rf gene (rf). When used together in multiplex PCR reactions the two sets of primer pairs allow for discrimination between RfRf (homozygous restorer), Rfrf (heterozygous restorer) and rfrf (non-restorer/sterile) genotypes.

Bands observed in multiplex PCR using primer sets C2 and Y5 in combination are:

- RfRf - one band (677 bp) is seen
- Rfrf - two bands (677 bp and 774 bp) are seen
- rfrf - one band (774 bp) is seen

A dot blot assay detection system based on the same two primer sets has also been developed, which works as follows:

- set up multiplex PCR using primer sets C2 and Y5 in combination;
- make two identical membranes by dotting PCR reaction onto membranes;
- probe the membranes with radioactive or non-radioactive probes. One membrane is probed with the 677 bp fragment linked to Rf, the second membrane is probed with the 774 bp fragment associated with rf; and
- compare the two membranes to determine OGU Rf genotype.

The system which is being utilized currently to genotype OGU populations is a combination of two SCAR markers, developed from RAPD bands. One marker C2 is linked to the restorer gene 'Rf' (developed using RAPD primer OPC2) and the other marker Y5 is linked to the absence of the restorer gene 'rf' (developed using RAPD primer OPY5). The combination of these two markers allows for the determination of OGU Rf genotype in individual plants, either 'RfRf', 'Rfrf', or 'rfrf'.

For each of the two SCAR markers there is a set of two standard PCR primers, one forward and one reverse, which were developed to amplify only the bands of interest (Table 2). One set of primers (C2) amplifies a band associated with the restorer gene, 'Rf', while the second set of primers (Y5) amplifies a band associated with the absence of the restorer gene 'rf'. These two sets of primers were developed so that they can both be utilized in the same PCR reaction. By using the two sets of primers in the same PCR reaction the problem of false negatives is eliminated, i.e. a failed PCR reaction will be obvious because no detectable result will be obtained by electrophoresis or by probing, either with radioactive or non-radioactive probes.

By using a multiplex format of PCR reactions, in this case two primer pairs in one reaction, at least one product should be formed. Absence of both products would signify failure of the PCR reaction. If the 'Rf' associated band is the only band amplified the individual plant is homozygous for the restorer gene (RfRf), if both the 'Rf' and 'rf' associated bands are amplified then the individual is heterozygous for the restorer gene (Rfrf). If only the 'rf' associated band is amplified then the individual is a homozygous non-restorer type (rfrf). If there are no bands amplified then the PCR reaction has failed for that sample and the reaction can be repeated. This procedure eliminates false negatives.

There are two main methods which can be utilized to visualize the results of the PCR amplifications. One method is to run the reaction products on an agarose gel and to stain with ethidium bromide to detect bands. While this method is adequate for small numbers of samples it is not conducive to large scale analysis of hundreds of samples. The pouring, loading, running of gels and staining/detection is very time consuming, labour intensive and costly.

This invention provides a dot blot system which allows for the rapid analysis of a large number of samples. In combination with a high throughput DNA extraction system, the dot blot system allows for the simultaneous testing of over 400 samples. With automation, the number of samples tested could be increased tremendously.

The dot blot assay involves "dotting" PCR products on duplicate blots and probing with the respective cloned RAPD band (discussed in more detail below).

The following Examples are presented as specific illustrations of the present invention. It should be understood, however, that the invention is not limited to the specific details set forth in the Examples.

Example 1 - Screening of Population 94CWN2133 to Identify RAPD Markers Linked to the Restorer Gene and, to Determine Which of These Markers Most Accurately Reflected the Genotype of the Plants

From seeds of the F2 population 94CWN2133, 369 plants were grown in greenhouse. Isozyme analysis was conducted on each plant using leaf tissue. In addition, plants were

scored for flowering phenotype, either fertile or sterile. Of these 369 plants, 175 were selected to be utilized in the linkage analysis to develop markers for the OGU restorer gene (Rf). Other plants were excluded from the study due to inconclusive isozyme score or because plants did not flower or were too late flowering. It was from these 175 plants that the two sets of bulk DNA were formed.

From bulk segregant analysis (see Materials and Methods, RAPD analysis) of the 500 RAPD primers screened, five [OPC2, OPH3, OPN20, OPH15, and OPF10] revealed amplification products that were present in fertile bulks and parent NW3002 (RfRf) and absent in sterile bulks, while four primers [OPG8, OPF6, OPY5, and OPG2] revealed amplification products that were present in the sterile bulks and parental sample, Bristol (rfrf), but were absent in the fertile bulks **(Table 1).**

Table 1. RAPD markers identified as being linked to OGU Rf (restored plants) or absence of OGU Rf (sterile plants)

RAPD markers	presence in restored plants	presence in sterile plants	RAPD Band size (bp)	SCAR marker developed?
OPC2	+	-	1139	yes
OPN20	+	-	1782	yes
OPY5	-	+	839	yes
OPH15	+	-	1345	yes
OPF10	+	-	883	yes
OPH3	+	-	613	yes
OPG2	-	+	1350*	no
OPG8	-	+	749	yes
OPF6	-	+	1240*	no

* bands were not sequenced - size estimated from gels

Individual F2 plants from population 94CWN2133, including those used to form bulks, were screened using the 9 RAPD markers which had been identified by bulked segregant analysis. The five markers associated with the fertile bulks co-segregated almost perfectly in the 175 individuals, i.e., in any individual either all five bands were present or all were absent. The same was observed for the four primers that were associated with the absence of the restorer gene. This indicated that recombination between these markers and the restorer gene is relatively rare.

Linkage analysis performed by using Mapmaker 3.0 (Whitehead Institute for Biomedical Research) indicated that OPC2, OPN20, OPH3 (markers associated with the Rf gene) and OPY5, OPG8, OPG2 and OPF6 (markers associated with the absence of the Rf gene) mapped the same locus, 1.4 cM from the Rf gene. The PGI II locus was mapped 1.5 cM on the opposite side of the restorer gene from the RAPD markers. Two other markers, OPH15 and OPF10, were also linked to the Rf gene but were distal, 0.6 cM and 1.7 cM, respectively, to the other RAPD markers (**Fig. 1**).

RAPD markers linked to the absence of the restorer gene were further tested on a set of ten regular canola varieties, i.e., normal cytoplasm and lacking the OGU Rf gene. Five of these varieties were winter *B. napus* and five were spring *B. napus*. Since none of the ten varieties contained the restorer gene it was expected that the four markers associated with the absence of the restorer gene should be present in all of these varieties. Two of the markers, OPG2 and OPY5, were found to be present in all ten varieties while the other two markers, OPF6 and OPG8, were inconsistent. It was concluded that OPY5 and OPG2 were more accurate for determining the absence of the restorer gene.

Example 2 - Conversion of RAPD Marker Bands to SCAR Markers and use of SCAR Markers to Determine Genotype

RAPD markers are somewhat unreliable and are not easily adapted to systems involving routine genotypic determinations. For this reason several SCAR markers, and primers for these markers, were developed from the RAPD markers. The selective use of SCAR primers in combination was used to determine plant genotype (RfRf, Rfrf, or rfrf) through detection of PCR products by gel electrophoresis or dot blot analysis.

Two SCAR markers, C2 and Y5, were found to work well together in combination.

In order to utilize the SCAR markers DNA was first extracted from the samples to be tested. Any DNA extraction system could be used, as is known to those skilled in the art. After DNA extraction, PCR reactions were set up using two sets of primer pairs in the same reaction. One set of primer pairs, set C2, amplified a 677 bp band associated with the restorer gene 'Rf'; the second set of primer pairs, set Y5, amplified a 774 bp band associated with the absence of the restorer gene 'rf'.

Table 2 shows the nucleotide sequence of the primer pairs involved. The PCR reaction mixture and techniques used for DNA amplification and visualization of the results were those described below in the Materials and Methods section.

Table 2 Nucleotide sequence of primers used to determine OGU Rf genotype

Primer	Sequence
C2 forward	GGGGAAGGAAGGAAGGACTC
C2 reverse	TCAGGTTCACACAGCAGCATA
Y5 forward	GAGCTGATGCACTGTAAGCAGT
Y5 reverse	GACGCCTAGATGTTGCCAGAAC

Example 3 - Visualization of PCR Results by Gel Electrophoresis on a 1.4% Agarose Gel

PCR products were loaded onto 1.4% agarose gels, either 1X TAE (0.04M Tris-acetate; 0.001M EDTA, pH 8.0) or 0.5X TBE (0.045M Tris-borate; 0.001M EDTA), containing 0.4 µg/ml ethidium bromide. Gels were run in the corresponding buffer for 4 hours at 80V and were then viewed under UV light. Photographs were taken to document the gels and sample genotypes were determined from these photos. For homozygous restorer individuals one band of approximately 677 bp was seen. For heterozygous individuals two bands were seen, one of about 677 bp and a second of about 774 bp. For individuals which did not contain any copies of the restorer gene one band of approximately 774 bp was seen.

Example 4 - Development of High Throughput Dot Blot Detection System

A high throughput dot blot detection system was developed. For blot analysis, the procedure described below in the Materials and Methods section was followed.

Example 5 - Determination of OGU Rf Genotype Using the Dot Blot Detection System

By comparing the autorads formed by the sets of identical membranes it was possible to correctly determine the OGU Rf genotype of the samples. For example, for a given sample on the membrane, if it showed a dot when probed by C2 but the duplicate sample on the sister membrane did not show a dot when probed with Y5 then the sample was homozygous restorer (RfRf). If one membrane showed a dot when probed with C2 and its sister membrane also showed a dot when probed with Y5 then the sample was heterozygous for the restorer gene (Rfrf). If one membrane showed a dot when probed with Y5 but its sister membrane did not show a dot when probed with C2 then the sample was homozygous non-restorer (rfrf). If neither membrane showed a dot then the PCR reaction had failed.

Example 6 - Testing of SCAR Markers Developed From RAPD Markers

In order to test the validity of the SCAR markers in terms of linkage to Rf gene, a second sample of 137 F2 plants developed from population 94CWN2133 were screened with the SCAR primer sets C2 and Y5. Results are summarized in **Table 3**:

Table 3. Numbers of plants in which SCAR markers were present (+) or absent (-) in restored (RR or Rr) and unrestored (rr) plants.

SCAR marker	Fertile, restored plants (106 plants)		Sterile, unrestored plants (36 plants)	
	+	-	+	-
C2	101	0[a]	1	35
Y5	76	25[a]	36	0

a - data was unavailable for five plants

These markers were further tested using a number of segregating populations from the spring and winter napus breeding programs. Table below illustrates the agreement of marker scores with PGI II and/or flowering scores (hence presence of Rf gene). For the purposes of illustration data for 10 plants per population are presented in the table, populations consisted of many more individuals and additional populations, other than those presented in the table, have also been tested. Test crosses were made on individuals from several populations in order to determine the accuracy of the SCAR markers. In almost all cases the expected segregation ratios were observed.

Table 4. Confirmation of markers

Line number	Plant number	Score using C2 (Rf) primer set	Score using Y5 (rf) primer set	OGU Rf Genotype	PGI II isozyme data	Flowering score
96SN1049 *sterile plants had been culled - tested using C2 and Y5 primers separately, run on gels	1	+	+	heterozygous	*Spring napus - PGI II scores not available	fertile
	4	+	+	heterozygous		fertile
	5	+	+	heterozygous		fertile
	6	+	+	heterozygous		fertile
	8	+	+	heterozygous		fertile
	9	+	+	heterozygous		fertile
	10	+	+	heterozygous		fertile
	11	+	-	homozygous		fertile
	13	+	+	heterozygous		fertile
	14	+	-	homozygous		fertile

96FNW1348-1 - tested using dot blot protocol	1	-	+	sterile	rr	flowering score unavailable
	2	+	+	heterozygous	Rr	
	3	+	+	heterozygous	Rr	
	4	-	+	sterile	rr	
	5	+	-	homozygous	RR	
	6	+	+	heterozygous	Rr	
	7	-	+	sterile	rr	
	8	+	+	heterozygous	Rr	
	9	-	+	sterile	rr	
	10	+	+	heterozygous	Rr	
94CWN2133 *second half of population - tested using dot blot protocol	1	-	+	sterile	rr	sterile
	2	+	+	heterozygous	Rr	fertile
	3	-	+	sterile	rr	sterile
	4	+	+	heterozygous	Rr	fertile
	5	+	+	heterozygous	Rr	fertile
	6	+	+	heterozygous	Rr	fertile
	7	+	+	heterozygous	Rr	fertile
	8	-	+	sterile	rr	sterile
	9	+	+	heterozygous	Rr	fertile
	10	+	+	heterozygous	Rr	fertile

Example 7 - Identification of AFLP Markers

Experiments to identify AFLP markers linked both to the OGU Rf restorer gene and to the absence of the restorer gene used bulked DNA samples derived from the second section of population 94CWN2133. Four bulks were used, two consisted of ten individuals each of homozygous restored plants while the other two bulks consisted of ten sterile individuals each. Screening of the population 94CWN2133 to identify AFLP markers linked to the restorer gene is done using techniques known to those skilled in the art. The AFLP method of amplification was conducted in accordance with the technique described in the Materials and Methods section of Vos, et al (1995).

Both AFLP and RAPD markers were screened on all 151 individuals from section 2 of population 94CWN2133 (see 'Plant material' under the 'AFLP Markers' section). Markers were mapped using Mapmaker (version 3.0) with a LOD threshold of 3.0 and a distance threshold of 80 Haldane cM. Using these parameters all markers were found to be linked on one linkage group. Marker orders were assigned using multipoint analysis and an initial map was constructed using the best log-likelihood scores as determined by using the 'ripple' command. However, in many cases, alternative marker orders were not statistically different from the best order. (See **Fig. 2**).

To date seventeen putative AFLP markers linked to the restorer gene have been identified along with eighteen possible markers linked to the absence of the restorer gene. Eight of the AFLP markers that have been developed (cloned, sequenced and mapped) are E36XM48AIII, E35XM62AV, E33XM47AI, E38XM60AI, E32XM50, E32XM59A, E32XM59B, and E33XM58. SCAR markers, and primers for these markers, are developed from these AFLP markers using techniques known to those skilled in the art.

The apparent discrepancy in the relative map position of RAPD markers in Fig. 1 *vs* Fig. 2 can reasonably be accounted for as follows: (i) use of different mapping populations and different size of populations, (ii) relative to Fig. 2, Fig. 1 was based on fewer markers, as well as fewer recombinations, (iii) map position of markers in Fig. 2 was based on a combination of linkage analysis and breakpoint analysis, that is, presence or absence of markers with successive markers on the Ogura Rf linkage group (**Table 5**). Given these differences, we have greater confidence in the map location of markers shown in Fig. 2. Only a few RAPD markers in Fig.1 (such as OPF6 and OPG8) were not

screened on second section of population 94CWN2133 and hence were not mapped in Fig 2.

Example 8 Determination of Breakpoints in Recombinant, Low Glucosinolate, OGU-Rf Winter and Spring *Brassica napus* Lines

The OGU-Rf restorer gene was transferred into canola, from *Raphanus sativus*, along with a PGI II isozyme marker. In winter napus lines the PGI II marker can be used for OGU genotype determination, as long as there have been no crossover events in which PGI II was lost. One of the greatest challenges with using the OGU system is that the presence of the restorer gene has been found to be linked to undesirably high levels of glucosinolates in the seed. The spring and winter napus breeding programs at Pioneer have developed several OGU-Rf lines which are fixed for the restorer gene and which contain acceptable (low) levels of glucosinolates (PCT International patent application no. PCT/CA95/01005 of Charne, David G., et al. filed December 19, 1997).

To gain further insight into the marker positions on the Rf locus, and to determine the breakpoints in recombinant low glucosinolate lines and hence identify recombinants with the smallest portion of *Raphanus* fragment, several recombinant, low glucosinolate, *B. napus*, restorer lines were screened using the RAPD and AFLP markers linked to the restorer gene. These lines were winter *napus* line NW1717M and NW1712, and spring *napus* lines NS3058 MO, NS3060 MO, 97SN7409, 97SN7416, 97SN7422, 97SN7423 and, 97SN7425. Control materials used included NW 3002 and Bristol. In addition, two restorer lines derived from crosses with NW 1717M were included: 97CWR150069 and 97CWR120046. Lines NW1717M and NS3060 MO were fixed restorer lines while the remaining lines were segregating for the restorer gene. In these segregating lines only homozygous restorer plants were tested. Markers that were absent in the respective recombinants would have been lost through recombination and would be indicative of the chromosome "breakpoint" in the recombinant line.

The following markers associated with the presence of the Ogu Rf gene were used: RAPD markers OPH3, OPN20, OPH15, OPF10, OPC2 and AFLP markers E36/M48, E35/M62, E33/M47, E38/M60, E32/M50, E32/M59A, E32/M59B, E33/M58, all of which indicate the presence of the Ogura restorer gene (Rf).

DNA was extracted from the lines of interest, PCR amplified and visualized. For RAPDs marker visualization was by electrophoresis on an agarose gel. For AFLPs visualization involved the use of an acrylamide gel.

Results are summarized in Tables 5 and 6 below (PCT International patent application no. PCT/CA95/01005 of Charne, David G., et al. filed December 19, 1997). The fertility of the lines of interest was confirmed by visual inspection of flower morphology. Glucosinolate analysis was conducted by TMS or HPLC and confirmed that the lines of interest had low glucosinolate content.

All markers were found to be present in lines NS3058 MO and NW1712. Therefore, these lines did not result from recombinations in which any of the OGU-Rf linked markers were lost.

Several of the markers were found to be absent in lines NS3060 MO and NW1717M, and two markers were found to be absent in each of lines 97SN7409, 97SN7416, 97SN7422, 97SN7423 and, 97SN7425. Therefore, these lines did result from recombinations in which breakpoints had occurred along the *Raphanus* chromosome segment near the restorer gene so that OGU-Rf linked markers were lost. This perhaps indicates that a smaller portion of the *Raphanus* chromosome segment had been transferred during these recombinations than had been transferred during the recombinations involving the NS3058 MO and NW1712 lines. Furthermore, the number of markers retained in NW1717M was less than in NS3060 MO, 97SN7409, 97SN7416, 97SN7422, 97SN7423 and 97SN7425, perhaps indicating that the *Raphanus* chromosome segment in NW1717M is smaller than that in NS3060 MO, 97SN7409, 97SN7416, 97SN7422, 97SN7423 and 97SN7425.

Table 5. Lines in which OGU-Rf linked markers were found to be present (+) or absent (-). NW3002 is a fixed restorer control and Bristol is a non-restorer control.

	NW3002	BRISTOL	NW 1717M	NS 3060MO	97SN 7409	97SN 7416	97SN 7422	97SN 7423	97SN 7425	CWR 150069	CWR 120046	NS3058 MO	NW1712
OPC2	+	-	+	+	+	+	+	+	+	+	+	+	+
OPF10	+	-	+	+	+	+	+	+	+	+	+	+	+
E38/M60	+	-	+	+	+	+	+	+	+	+	+	+	+
E35/M62	+	-	+	+	+	+	+	+	+	+	+	+	+
OPN20	+	-	-	+	+	+	+	+	+	-	-	+	+
E33/M47	+	-	-	+	+	+	+	+	+	-	-	+	+
E32/M50	+	-	-	+	+	+	+	+	+	-	-	+	+
OPH3	+	-	-	-	+	+	+	+	+	-	-	+	+
OPH15	+	-	-	-	+	+	+	+	+	-	-	+	+
E33/M58	+	-	-	-	+	+	+	+	+	-	-	+	+
E32/M59A	+	-	-	-	+	+	+	+	+	-	-	+	+
E36/M48	+	-	-	-	-	-	-	-	-	-	-	+	+
E32/M59B	+	-	-	-	-	-	-	-	-	-	-	+	+
PGI II	+	-	-	na	na	Na	na	na	na	na	na	na	na

na = data not available

Table 6 : Confirmation of low glucosinolate content and Rf stability

UPDATED INFORMATION – GLUCOSINOLATE CONTENT AND Rf STABILITY							
CVN Code (New)	BLN Code (Previous)	Total Glucosinolates (micromol/g by TMS) 96/97 (Chile)	1997 (Ontario)	No. of fertile and sterile plants in a sample (Ontario, 1997) * Inbred Fertile	Sterile	Test Cross Fertile	Sterile
NS3059	97SN-1649	11.45	11.32	600	0	38	2
NS3060	97SN-1650	14.40	14.01	600	0	42	0
CVN Code (New)	BLN Code (Previous)	Total Glucosinolates (micromol/g by HPLC) 1996/97 (France)		No. of fertile and sterile plants in a sample (France, 1997) * Inbred Fertile	Sterile	Test Cross Fertile	Sterile
NW1717	96FNW-1822-2	8.73		2,000	0	321	1
	96FNW-1822-5	10.16		2,000	0	412	1
	96FNW-1822-7	8.14		2,000	0	420	0
	96FNW-1822-8	9.82		2,000	0	346	2
NW1712	96FNW-1348-6	14.71		2,000	0	375	2

*Fertile/sterile classification by visual inspection of flower morphology

Materials and Methods

RAPD Markers

Plant material: Plant material used was the winter *B. napus* F_2 OGU restorer population 94CWN2133, and its parents NW3002 and Bristol, which were obtained from the winter canola breeding group at Pioneer Hi-Bred. Plants had been grown to the four leaf stage, vernalized for 12 weeks at 4°C, and then grown in the greenhouse. Plants from this population were screened for the presence of the restorer gene using the *Pgi-2* isozyme marker (Delourme and Eber, 1992). Isozyme analysis was done at Pioneer's Johnston, Iowa, electrophoresis laboratory. Based on isozyme pattern plants from this population were classified according to genotype, either homozygous restorer (RfRf), heterozygous restorer (Rfrf), or unrestored (rfrf). Individual plants were also scored as male fertile or sterile based on flower phenotype, i.e.. whether pollen/anther were normal or non-functional.

DNA Extraction: DNA was extracted from lyophilized ground leaf tissue using a modified CTAB extraction protocol (Doyle and Doyle, 1990). For each sample, 0.3g of ground leaf tissue was placed into tubes containing 10ml of CTAB buffer (0.1M Tris, 0.7M NaCl, 10mM EDTA, 27mM CTAB, 1% B-mercapotethanol). Tubes were incubated at 60°C for 1 hour. Chloroform:isoamyl alcohol, 24:1, (5 ml) was added and the blended suspensions were centrifuged at 6,000 rpm for 5 min in a Beckman J2-HS centrifuge. Supernatants were recovered and a second chloroform/isoamyl extraction was carried out. Supernatants were then transferred to new tubes and 8ml of isopropanol was added to precipitate the DNA. After centrifuging for 5 min. at 5000 rpm the supernatants were poured off and 4 mls of 76% EtOH, 0.2M NaOAc was added. This solution was left for 20 min. before being poured off. The pellets were washed in 76% EtOH, 10mM NH_4OAc and then resuspended in 9 mls of TE buffer (pH 8.0). Once DNA was resuspended an additional two chloroform/isoamyl extractions were carried out as needed. After the last chloroform/isoamyl extraction 7.0 mls of 20% PEG, 2.5M NaCl was added to the supernatants, the two solutions were mixed and tubes were placed on ice for 1 hour. After 1 hour tubes were centrifuged at 8,000 rpm for 10 min. at 4°C. Supernatants were discarded and pellets were washed in 70% chilled ethanol prior to being resuspended in 0.5 ml TE containing 50 ug/ml RNAse A. DNA concentrations were determined by fluorescence in the presence of bisbenzimide (Hoescht dye 33258) using a TKO 100 fluorometer (Hoefer).

RAPD analysis: The Bulk Segregant Analysis (BSA) method (Michelmore et al. 1991) was used for the identification of putative markers linked to the Ogura Rf gene.

DNA aliquots of 2 ug each were collected from homozygous fertile individuals and from homozygous sterile individuals. These aliquots were combined to form four separate DNA pools, two pools of homozygous fertile individuals (RfRf) and two pools of homozygous sterile (rfrf) individuals. The number of individuals in each bulk was either 17 or 18.

The DNA "bulks" along with DNA from parental lines, NW3002 (homozygous restorer) and Bristol (homozygous non-restorer), were diluted to 25 ng/µl. RAPD markers were tested for their ability to detect polymorphism between the two types of pooled DNA "bulks", as well as, between the two parents. Primer kits A through Y from Operon

Technologies, consisting of a total of 500 oligonucleotides, were used as primers. One µl of the diluted DNA was used as template in a final reaction volume of 15µl. The PCR reaction solution contained 1X PCR Buffer II (Perkin Elmer), 2.5 mM $MgCl_2$, 200 µM each of dATP, dCTP, dGTP, and dTTP, 0.4 µM Operon primer, 0.375 Units - of *Taq* polymerase (Perkin Elmer). DNA amplification was performed in a Perkin Elmer Geneamp PCR System 9600 thermocycler programmed as follows: 2 min. at 94°C for 1 cycle; 5 sec. at 94°C, 30 sec. at 36°C, and 1 min. at 72°C for 35 cycles; 5 min. at 72°C for 1 cycle; hold at 4°C. Amplification products were separated by gel electrophoresis on 1.4% agarose gels and visualized by ethidium bromide staining. Primers detecting polymorphisms were then tested on all individuals from population 94CWN2133 to determine which primers gave polymorphic bands linked either to the presence or absence of the restorer gene. Gels for this analysis were either 1.4% agarose or 2% agarose (1% Metaphore and 1% NuSieve 3:1; FMC BioProducts).

Conversion to SCAR Primers: Bands which showed linkage to either the restorer gene or the absence of the restorer gene were cloned using the TA Cloning Kit (Invitrogen) and were sequenced. Based on the sequence of the cloned bands oligonucleotides were designed such that the fragment of interest could be amplified by PCR, using more reproducible reaction conditions. PCR products or marker bands developed this way are referred to as Sequence Characterized Amplification Regions or SCARS. The primers were designed with similar annealing temperatures so that they might be used together in multiplex PCR reactions. These SCAR primers were tested against individuals from the 94CWN2133 segregating population to determine if they accurately reflected the genotype of the plants as characterized by the RAPD primers.

The PCR reaction mixture (20.0 µl) for testing the SCAR marker primers contained 25 ng genomic DNA, 1X PCR Buffer II (Perkin Elmer), 1.5 mM $MgCl_2$, 250 µM each of dATP, dCTP, dGTP, and dTTP, 22.5 pmoles each of forward and reverse primers, 0.5 U of *Taq* polymerase (Perkin Elmer). DNA amplification was performed in a Perkin Elmer Geneamp PCR System 9600 thermocycler programmed as follows: 2 min. at 94°C for 1 cycle; 1 min. at 94°C, 1 min. at 60°C, and 1 min. at 72°C for 35 cycles; 5 min. at 72°C for 1 cycle; hold at 4°C. Amplification products were visualized on 1.4% agarose gels.

From a total of nine SCAR markers developed, one linked to the restorer gene (marker C2) and a second linked to the absence of the restorer gene (marker Y5), gave very reproducible and clear bands when tested together in multiplex PCR reactions. SCAR markers H3 and H15 (linked to the restorer gene) and G8 (linked to the absence of the restorer gene) did not give reproducible, clear bands when used in multiplex PCR reactions. The PCR reaction mixture (20.0 ul) which was found to give the best results for multiplexing these two primer sets was: 10-25 ng genomic DNA, 1X PCR Buffer II (Perkin Elmer), 1.5 mM $MgCl_2$, 250 µM each of dATP, dCTP, dGTP, and dTTP, 19.5 pmoles C2 primers (forward and reverse in equal mix), 25.5 pmoles Y5 primers (forward and reverse in equal mix), 0.5 U of *Taq* polymerase (Perkin Elmer). DNA amplification was performed in a Perkin Elmer Geneamp PCR System 9600 thermocycler programmed as follows: 2 min. at 94°C for 1 cycle; 1 min. at 94°C, 1 min. at 60°C, and 1 min. at 72°C for 35 cycles; 5 min. at 72°C for 1 cycle; hold at 4°C. After PCR, results were visualized by gel electrophoresis on a 1.4% agarose gel or, results were visualized by means of a dot blot assay (as described earlier).

Development of a Dot Blot System: A dot blot detection system for visualizing the results of multiplex PCR reactions using the two SCAR primer sets C2 and Y5 was developed. This same system could be utilized using any other combination of primers which lend themselves to multiplex PCR.

For dot blot analysis, 5 µl of PCR product from the multiplex PCR reactions were dotted onto each of two Hybond™ N+ nylon membranes. The membranes were then removed and placed DNA side up on blotting pad saturated with 0.6M NaCl, 0.4M NaOH for 2 min, then transferred to blotting pad saturated with 0.5M Tris (pH 7.5), 1.5M NaCl for 10 min. Samples were bound to the membranes by baking for 30 min. to 1 hour at 80°C. Of the two identical membranes, one was probed with the 'Rf' associated probe (C2) and the other probed with the 'rf' associated probe (Y5).

Prehybridization was done in a Hybaid™ oven at 65°C for 2 hours to overnight. Prehybridization/hybridization solution consisted of 1% SDS, 10% dextran sulphate sodium salt 500K, 5X SSPE (0.9M NaCl, 50mM $Na_2HPO_4.7H_2O$, 5mM EDTA, pH 7.7), 10X Denhardts (0.2% BSA fraction V, 0.2% ficoll 400K, 0.2% polyvinylpyrolidone 360K). DNA probes used for hybridization were fragments amplified from the plasmid

clones obtained from cloning of original RAPD marker bands, using the same primer sets (C2 and Y5). Amplified probes were gel purified and isolated using a GENECLEAN kit (BIO 101, Inc.). During prehybridization, probes were labeled with ^{32}P using the Amersham multiprime DNA labeling system (RPN.1600Z) and purified using the protocols for NICK® columns (Pharmacia Biotech). After prehybridization, labeled probes were added to the prehybridization solution and membranes were hybridized for 1 hour to overnight. From the sets of two identical membranes, one membrane was probed with the C2 probe (Rf associated) while its sister membrane was probed with the Y5 probe which is associated with the absence of the restorer gene. After hybridization membranes were washed once at 60°C in 2 X SSC, 0.1% SDS for 20 min followed by two washes in 0.1 X SSC, 0.1% SDS for 30 min. at 60°C. Dot blots were exposed to Amersham Hyperfilm™-MP for periods varying from 4 hours to overnight.

The OGU Rf genotype of the samples was determined by comparing the autorads formed by the sets of identical membranes. For example, for a given sample on the membrane, if it showed a dot when probed by C2 but the duplicate sample on the sister membrane did not show a dot when probed with Y5 then the sample is homozygous restorer (RfRf). If one membrane showed a dot when probed with C2 and its sister membrane also showed a dot when probed with Y5 then the sample is heterozygous for the restorer gene (Rfrf). If one membrane showed a dot when probed with Y5 but its sister membrane did not show a dot when probed with C2 then the sample is homozygous non-restorer (rfrf). If neither membrane showed a dot then the PCR reaction had failed.

AFLP Markers

Plant material: Plant material used for AFLP analysis was a winter *B. napus* population which was derived from the winter *B. napus* OGU population, 94CWN2133, used for RAPD analysis. The population used for AFLP analysis was derived by crossing homozygous restorer plants from population 94CWN2133 with sterile plants from the same population. The resulting heterozygous offspring were then selfed resulting in a population segregating for the restorer gene. This population is referred to as section 2 of population 94CWN2133. Plants from section 2 of population 94CWN2133 were

vernalized, screened with the *Pgi-2* isozyme marker, and scored for flowering phenotype following the same protocols used for the original 94CWN2133 population. The second section of population 94CWN2133 consisted of 151 individuals. Several of these individuals were eliminated due to lack of flowering or PGI II scores, making the final population 137 individuals.

DNA Extraction: DNA was extracted following the same protocol used for the original 94CWN2133 population, a modified CTAB extraction protocol (Doyle and Doyle, 1990). For section two of population 94CWN2133 0.1g of ground, lyophilized leaf tissue was used for extraction. Following the final chloroform/isoamyl extraction DNA was precipitated with 1/10 volume of 3M NaOAc and 2/3 volume propanol instead of with 20%PEG, 2.5M NaCl . Tubes were inverted to mix and then centrifuged at 8,000 rpm for 10min. at 4°C prior to washing with 70% ethanol and resuspending in TE.

AFLP analysis: As with the RAPD analysis, Bulk Segregant Analysis (Michelmore et al. 1991) was used for the identification of putative AFLP markers linked to the Ogura Rf gene. To form bulked DNA samples with concentrations of 50 ng/ul, DNA aliquots of 5 ng each were collected from homozygous fertile individuals and from homozygous sterile individuals. The 5 ng aliquots were combined to form two homozygous fertile (RfRf) pools and two homozygous sterile (rfrf) pools. The number of individuals in each pool/bulk was 10.

DNA bulks and parental samples, NW3002 and Bristol, were screened using the AFLP protocol (Vos et al. 1995). DNA was digested using EcoRI and MseI enzymes and first step amplification was performed using AFLP primers having a single selective nucleotide. For first step amplification 50ul PCR reactions were set up containing 75 ng of both AFLP primers, 5ul of DNA template (1:10 dilution of digestion/ligation mix), 1U *Taq* polymerase (Perkin Elmer), 1X PCR buffer (Perkin Elmer) and 1.25 mM of all four dNTPs. After PCR, first step amplification reaction mixes were diluted 20-fold before being used as templates for the second amplification reaction. Reactions for labeling of EcoRI primers for the second amplification were performed in 1X One-Phor-All buffer PLUS (Pharmacia Biotech).

After the second amplification an equal volume of formamide dye (98% formamide, 10mM EDTA pH 8.0, and bromophenol blue and xylene cyanol as tracking dyes) was added and the resulting mixes were then heated for 3min. at 90°C and cooled on ice. Each sample (3-5ul depending on combs used) was loaded on a 4.5% denaturing polyacrylamide gel. Gels were prepared using a solution containing 4.5% acrylamide/Bis Solution 19:1 (Bio-Rad Laboratories), 7.5 M urea, 50mM Tris, 50mM Boric acid, 1mM EDTA. A volume of 120 ml of gel solution was degassed in a desiccator attached to a vacuum source for 20 min. To this solution 100 ul TEMED and 500 ul 10% APS were added and gels were cast using a Sequi-Gen®GT Nucleic Acid Electrophoresis Cell (Bio-Rad Laboratories). The bottom buffer tray contained 1.25 M sodium acetate, 100mM Tris, 100mM Boric acid, 2mM EDTA and, 100mM Tris, 100mM Boric acid, 2mM EDTA was used as a running buffer. Electrophoresis was performed at 120W, 45°C for approximately 2 hours. After electrophoresis gels were dried for 1 hr at 80°C in a dual temperature slab gel dryer (Bio-Rad Laboratories) and were exposed to film (Hyperfilm™-MP, Amersham) for 4-7 days.

Polymorphic bands were identified as being putative markers for the Ogu restorer gene. These putative markers are then screened against the individuals from section 2 of population 94CWN2133 to determine how closely linked the markers are to the restorer gene.

Conversion of AFLP Markers to SCAR Markers: AFLP bands showing linkage to the restorer gene are cloned and sequenced. Based on the sequence information SCAR primers are developed to amplify the bands of interest. These SCAR primers are tested on section two of population 94CNW2133 to determine if they accurately reflect the genotype of the individuals. The protocol used for testing of SCAR primers is the same as that used to test SCAR primers developed from RAPD bands. These primers are designed with similar annealing temperatures to those which were developed from RAPD bands so that the primers might be used together in multiplex PCR reactions.

All publications, patents and patent applications are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

The present invention has been described in detail and with particular reference to the preferred embodiments, however, it will be understood by one having ordinary skill in the art that changes can be made thereto without departing from the spirit and scope thereof.

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We claim:

1. A method for determining the genotype of *Brassica* germplasm for the Ogura (Rf) restorer gene in a *Brassica* breeding program, comprising the steps of:
   - amplifying the *Brassica* germplasm for the Ogura (rf) restorer gene using at least one primer, and
   - determining the genotype using at least two nucleic acid markers, wherein one marker indicates the presence of the OGU Rf gene (Rf) and the other marker indicates the absence of the OGU Rf gene (rf).
2. The method of claim 1, wherein the markers are selected from a group consisting of SCAR markers, RAPD markers and AFLP markers.
3. The method of claim 1, wherein the marker indicating the absence of the OGU Rf gene (rf) is a SCAR marker selected from a group consisting of
   - Y5, represented by SEQ ID NO: 1,
   - a marker having partial homology to that sequence, and
   - any other marker selected from a sequence of the RAPD band from which that marker is derived.
4. The method of claim 1, wherein the marker indicating the presence of the OGU Rf gene (Rf) is a SCAR marker selected from a group consisting of
   - C2, represented by SEQ ID NO: 2,
   - N20, represented by SEQ ID NO: 3,
   - F10, represented by SEQ ID NO: 4,
   - a marker having partial homology to one of those sequences, and
   - any other marker selected from a sequence of the RAPD band from which these markers are derived.
5. The method of claim 1, wherein the markers are SCAR markers and the marker indicating the presence of the OGU Rf gene (Rf) is C2 having the sequence set out in SEQ ID NO 2, or a marker having partial homology to C2, and the marker indicating the absence of the OGU Rf gene (rf) is Y5 having the sequence set out in SEQ ID NO 1, or a marker having partial homology to Y5.

6. The method of claim 1, wherein the marker indicating the absence of the OGU Rf gene (rf) is a RAPD marker selected from a group consisting of

- OPY5, represented by SEQ ID NO: 5,
- OPG8, represented by SEQ ID NO: 6,
- a marker having partial homology to one of those sequences, and
- any other marker selected from a sequence of the band from which these markers are derived.

7. The method of claim 1, wherein the marker indicating the presence of the OGU Rf gene (Rf) is a RAPD marker selected from a group consisting of

- OPC2, represented by SEQ ID NO: 7,
- OPN20, represented by SEQ ID NO: 8,
- OPH3, represented by SEQ ID NO: 9,
- OPF10, represented by SEQ ID NO: 10,
- OPH15, represented by SEQ ID NO: 11,
- a marker having partial homology to one of those sequences, and
- any other marker selected from a sequence of the band from which these markers are derived.

8. The method of claim 1, wherein the marker indicating the presence of the OGU Rf gene (Rf) is a AFLP marker selected from a group consisting of

- E36XM48AIII, represented by SEQ ID NO: 12,
- E35XM62AV, represented by SEQ ID NO: 13,
- E33XM47AI, represented by SEQ ID NO: 14,
- E38XM60AI, represented by SEQ ID NO: 15,
- E32XM50, represented by SEQ ID NO: 16,
- E32XM59A, represented by SEQ ID NO: 17,
- E32XM59B, represented by SEQ ID NO: 18,
- E33XM58, represented by SEQ ID NO: 19,
- a marker having partial homology to one of those sequences, and
- any other marker selected from a sequence of the band from which these markers are derived.

9. The method of claim 1, wherein the amplification step comprises at least one polymerase chain reaction (PCR) and primers for PCR are selected from the following group of primers, or from primers having partial homology to the following group of primers:

C2 forward	GGGGAAGGAAGGAAGGACTC
C2 reverse	TCAGGTTCACACAGCAGCATA
Y5 forward	GAGCTGATGCACTGTAAGCAGT
Y5 reverse	GACGCCTAGATGTTGCCAGAAC

10. The method of claim 9, wherein the method comprises a multiplex polymerase chain reaction (PCR) using all of the primers in a single reaction.

11. The method of claim 1, wherein the step of determining the genotype comprises the use of a dot blot assay to detect the genotype.

12. The method of claim 1, wherein the amplification step comprises using primer sets C2 and Y5 in combination in a multiplex polymerase chain reaction (PCR), and the step of determining the genotype comprises

- dotting PCR reaction onto two identical membranes,
- probing the membranes with probes, wherein one membrane is probed with a probe which hybridizes to a marker indicating the presence of Rf and the second membrane is probed with a probe which hybridizes to a marker indicating the absence of rf, and
- comparing the two membranes to determine the genotype of *Brassica* germplasm for the Ogura restorer.

13. The method of claim 12, wherein the marker indicating the presence of Rf is about 677 base pairs and the marker indicating the absence of rf is about 774 base pairs.

14. The method of claim 1, wherein the amplification step comprises using primer sets C2 and Y5 in combination in a multiplex polymerase chain reaction (PCR), and the step of determining the genotype comprises

- running out the products of PCR on an electrophoresis gel, wherein the reaction products are from a group consisting of:
    - one band (about 677 bp), indicating a genotype of RfRf,

- two bands (about 677 bp and about 774 bp), indicating a genotype of Rfrf, and
- one band (about 774 bp), indicating a genotype of rfrf, and

- reading the gel to determine the genotype of *Brassica* germplasm for the Ogura restorer.

15. The method of claim 1, wherein the *Brassica* germplasm is selected from a group consisting of winter and spring *Brassica napus, Brassica rapa* and *Brassica juncea.*

16. A homozygous locus associated with the presence of the Ogura (RfRf) restorer gene, said locus mapping to at least one of the following markers:

- C2, represented by SEQ ID NO: 2,
- N20, represented by SEQ ID NO: 3,
- F10, represented by SEQ ID NO: 4,
- a marker having partial homology to one of those sequences, and
- any other marker selected from a sequence of the RAPD band from which these markers are derived.

17. A homozygous locus associated with the presence of the Ogura (RfRf) restorer gene, said locus mapping to at least one of the following markers:

- OPC2, represented by SEQ ID NO: 7,
- OPN20, represented by SEQ ID NO: 8,
- OPH3, represented by SEQ ID NO: 9
- OPF10, represented by SEQ ID NO: 10,
- OPH15, represented by SEQ ID NO: 11,
- E36XM48AIII, represented by SEQ ID NO: 12,
- E35XM62AV, represented by SEQ ID NO: 13,
- E33XM47AI, represented by SEQ ID NO: 14,
- E38XM60AI, represented by SEQ ID NO: 15,
- E32XM50, represented by SEQ ID NO: 16,
- E32XM59A, represented by SEQ ID NO: 17,
- E32XM59B, represented by SEQ ID NO: 18,
- E33XM58, represented by SEQ ID NO: 19,
- a marker having partial homology to one of those sequences, and

- any other marker selected from a sequence of the band from which these markers are derived.

18. A homozygous locus associated with the absence of the Ogura (rfrf) restorer gene, said locus mapping to one of the following markers:
    - Y5, represented by SEQ ID NO: 1,
    - a marker having partial homology to one of those sequences, and
    - any other marker selected from a sequence of the RAPD band from which these markers are derived.

19. A homozygous locus associated with the absence of the Ogura (rfrf) restorer gene, said locus mapping to one of the following markers:
    - OPY5, represented by SEQ ID NO: 5,
    - OPG8, represented by SEQ ID NO. 6
    - a marker having partial homology to one of those sequences, and
    - any other marker selected from a sequence of the band from which these markers are derived.

20. A heterozygous locus associated with the presence of the Ogura (Rfrf) restorer gene, said locus mapping to one of the markers of claim 16 or 17 and one of the markers of claim 18 or 19.

21. A combination of markers for determining the genotype of *Brassica* germplasm for the Ogura (Rf) restorer gene, wherein at least one marker indicates the presence of the Ogura (Rf) restorer gene and at least one marker indicates the absence of the Ogura (rf) restorer gene.

22. A combination of markers for determining the genotype of *Brassica* germplasm for the Ogura (Rf) restorer gene, comprising a first set of nucleic acid markers selected from the group consisting of
    - C2, represented by SEQ ID NO: 2,
    - N20, represented by SEQ ID NO: 3,
    - F10, represented by SEQ ID NO: 4,
    - a marker having partial homology to one of those sequences, and
    - any other marker selected from a sequence of the RAPD band from which these markers are derived,

and markers comprising a second set of nucleic acid markers selected from a group consisting of

- Y5, represented by SEQ ID NO: 1
- a marker having partial homology to one of those sequences, and
- any other marker selected from a sequence of the RAPD band from which these markers are derived.

23. A combination of markers for determining the genotype of *Brassica* germplasm for the Ogura (Rf) restorer gene, comprising a first set of nucleic acid markers selected from the group consisting of

- OPC2, represented by SEQ ID NO: 7,
- OPN20, represented by SEQ ID NO: 8,
- OPH3, represented by SEQ ID NO: 9,
- OPF10, represented by SEQ ID NO: 10,
- OPH15, represented by SEQ ID NO: 11,
- E36XM48AIII, represented by SEQ ID NO: 12,
- E35XM62AV, represented by SEQ ID NO: 13,
- E33XM47AI, represented by SEQ ID NO: 14,
- E38XM60AI, represented by SEQ ID NO: 15,
- E32XM50, represented by SEQ ID NO: 16,
- E32XM59A, represented by SEQ ID NO: 17,
- E32XM59B, represented by SEQ ID NO: 18,
- E33XM58, represented by SEQ ID NO: 19,
- a marker having partial homology to one of those sequences, and
- any other marker selected from a sequence of the band from which these markers are derived,

and markers comprising a second set of nucleic acid markers selected from a group consisting of

- OPY5, represented by SEQ ID NO: 5,
- OPG8, represented by SEQ ID NO. 6,
- a marker having partial homology to one of those sequences, and
- any other marker selected from a sequence of the band from which these markers are derived.

24. A SCAR marker for determining the presence of the Ogura (Rf) restorer gene in the genotype of *Brassica* germplasm, comprising a nucleic acid marker selected from the group consisting of

- C2, represented by SEQ ID NO: 2,
- N20, represented by SEQ ID NO: 3,
- F10, represented by SEQ ID NO: 4,
- a marker having partial homology to one of those sequences, and
- any other marker selected from a sequence of the RAPD band from which these markers are derived.

25. A SCAR marker for determining the absence of the Ogura (Rf) restorer gene in the genotype of *Brassica* germplasm, comprising a nucleic acid marker selected from the group consisting of

- Y5, represented by SEQ ID NO: 1,
- a marker having partial homology to one of those sequences, and
- any other marker selected from a sequence of the RAPD band from which these markers are derived.

26. A RAPD marker for determining the presence of the Ogura (Rf) restorer gene in the genotype of *Brassica* germplasm, comprising a nucleic acid marker selected from the group consisting of

- OPN20, represented by SEQ ID NO: 8,
- OPH3, represented by SEQ ID NO: 9,
- OPF10, represented by SEQ ID NO: 10,
- OPH15, represented by SEQ ID NO: 11,
- a marker having partial homology to one of those sequences, and
- any other marker selected from a sequence of the band from which these markers are derived.

27. An AFLP marker for determining the presence of the Ogura (Rf) restorer gene in the genotype of *Brassica* germplasm, comprising a nucleic acid marker selected from the group consisting of

- E36XM48AIII, represented by SEQ ID NO: 12,
- E35XM62AV, represented by SEQ ID NO: 13,
- E33XM47AI, represented by SEQ ID NO: 14,

- E38XM60AI, represented by SEQ ID NO: 15,
- E32XM50, represented by SEQ ID NO: 16,
- E32XM59A, represented by SEQ ID NO: 17,
- E32XM59B, represented by SEQ ID NO: 18,
- E33XM58, represented by SEQ ID NO: 19,
- a marker having partial homology to one of those sequences, and
- any other marker selected from a sequence of the band from which these markers are derived.

28. A combination of primers for use in a polymerase chain reaction (PCR) to determine the genotype of Brassica germplasm for the Ogura (Rf) restorer gene, comprising primers selected from the following group of primers, from primers having at least partial homology to the following group of primers, from primers which anneal to a sequence from which these primers are derived, or from primers derived from any portion of the RAPD fragment or amplified RAPD fragment from which the following primers are derived:

C2 forward	GGGGAAGGAAGGAAGGACTC
C2 reverse	TCAGGTTCACACAGCAGCATA
Y5 forward	GAGCTGATGCACTGTAAGCAGT
Y5 reverse	GACGCCTAGATGTTGCCAGAAC

29. A primer kit, comprising the primers of claim 28.

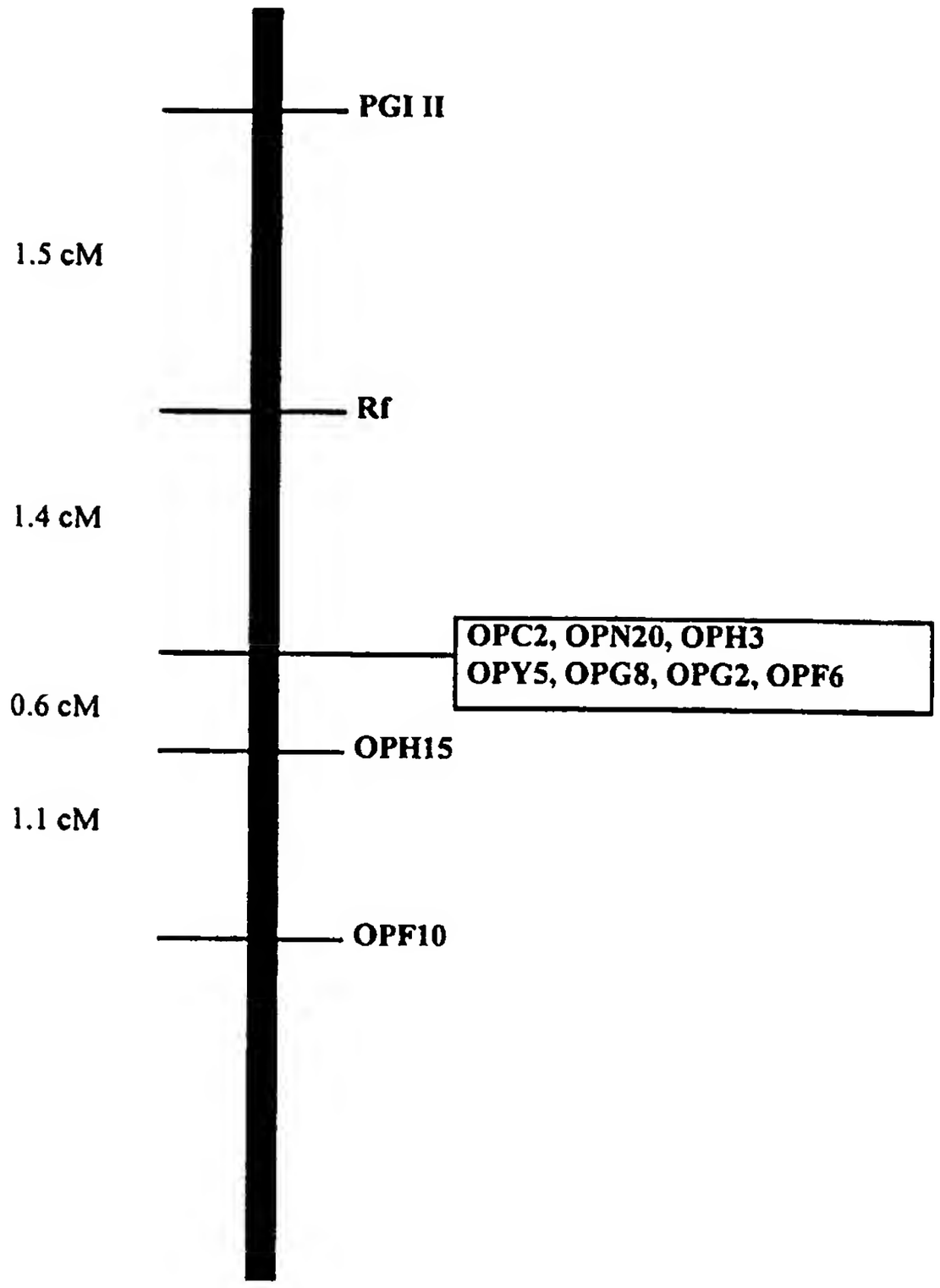
30. A system for determining the genotype of *Brassica* germplasm for the Ogura (Rf) restorer gene, comprising the steps of:

- amplifying the genotype in a single multiplex polymerase chain reaction (PCR), using primers selected from the following group of primers, from primers having at least partial homology to the following group of primers,

from primers which are complementary to these primers, from primers which anneal to a sequence from which these primers are derived, or from primers derived from any portion of the RAPD fragment or amplified RAPD fragment from which the following primers are derived:

C2 forward	GGGGAAGGAAGGAAGGACTC
C2 reverse	TCAGGTTCACACAGCAGCATA
Y5 forward	GAGCTGATGCACTGTAAGCAGT
Y5 reverse	GACGCCTAGATGTTGCCAGAAC

- determining the genotype using a combination of nucleic acid markers, wherein the markers are selected from a group consisting of: a C2 marker indicating the presence of the OGU Rf gene (Rf), or markers having at least partial homology with the C2 marker, and a Y5 marker indicating the absence of the OGU Rf gene (rf), or markers having at least partial homology with the Y5 marker.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Rf
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OPC2, OPN20, OPH3
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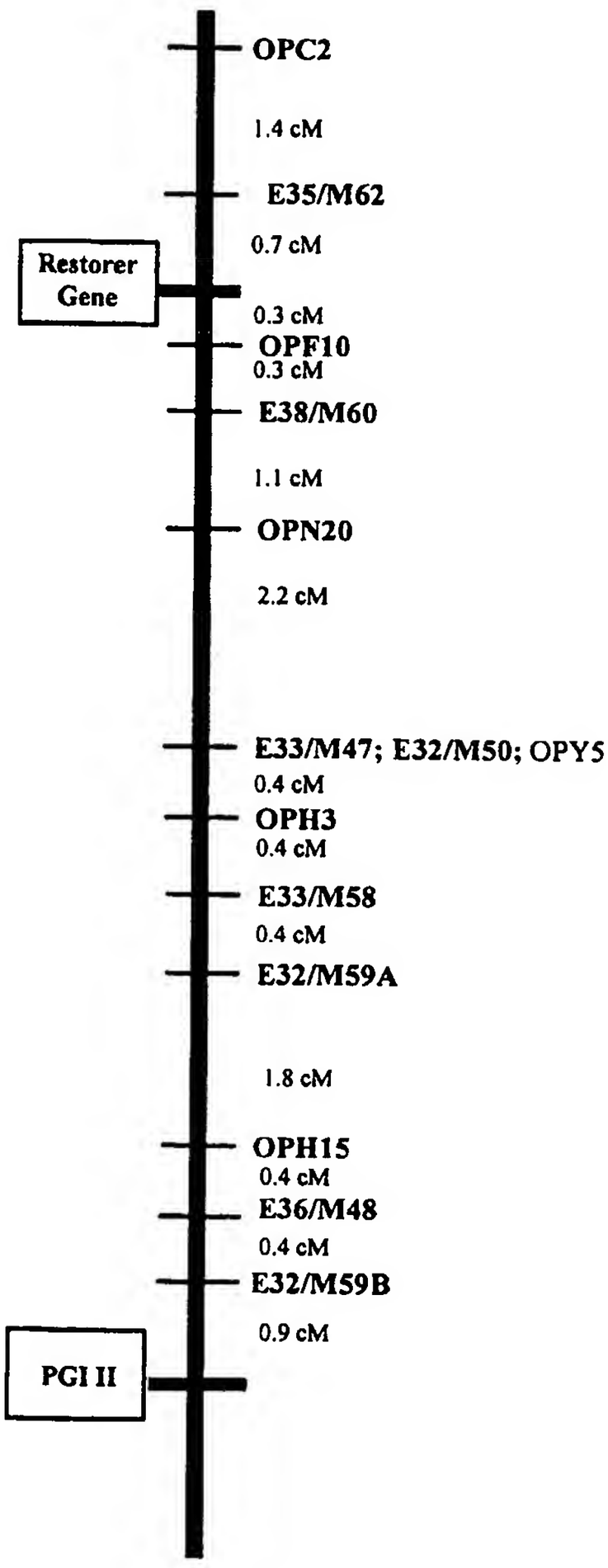
OPH15
1.1 cM
OPF10

Figure 1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Restorer Gene
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E38/M60
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OPN20
2.2 cM
E33/M47; E32/M50; OPY5
0.4 cM
OPH3
0.4 cM
E33/M58
0.4 cM
E32/M59A
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OPH15
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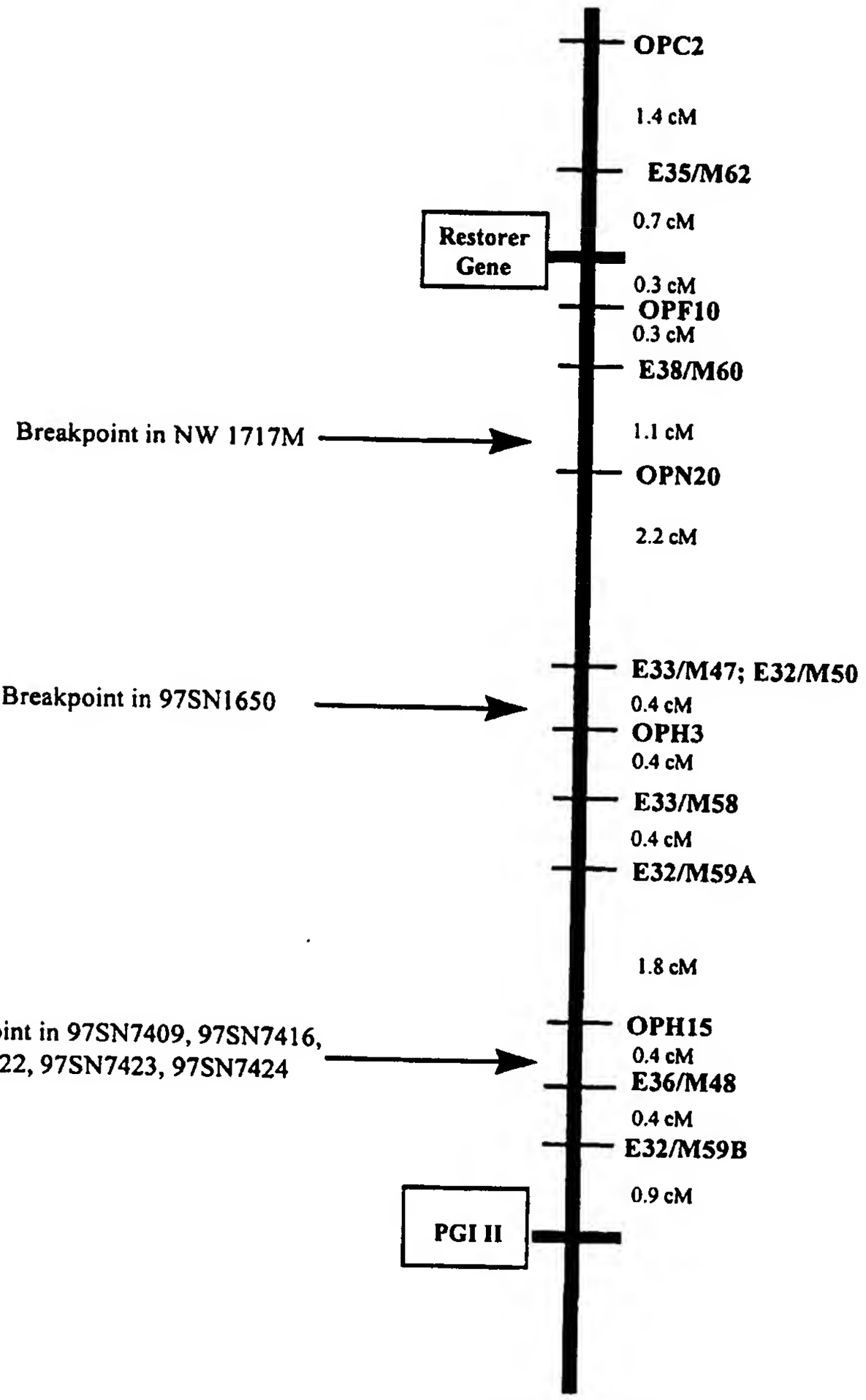
E36/M48
0.4 cM
E32/M59B
0.9 cM
PGI II

Figure 2


OPC2
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E35/M62
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Restorer Gene
0.3 cM
OPF10
0.3 cM
E38/M60
Breakpoint in NW 1717M
1.1 cM
OPN20
2.2 cM
E33/M47; E32/M50
Breakpoint in 97SN1650
0.4 cM
OPH3
0.4 cM
E33/M58
0.4 cM
E32/M59A
1.8 cM
OPH15
Breakpoint in 97SN7409, 97SN7416, 97SN7422, 97SN7423, 97SN7424
0.4 cM
E36/M48
0.4 cM
E32/M59B
0.9 cM
PGI II

Figure 3

INTERNATIONAL SEARCH REPORT

:national Application No
PCT/CA 98/00511

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 A01H1/04

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q C12N A01H

Documentation searched other than minimumdocumentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 97 02737 A (ZENCO NO 4 LIMITED) 30 January 1997	1,2,4,7, 11-15, 21-23, 26,28-30
X	see whole document, especially table 7 and pages 20-23.	17,20

A	DELOURME R ET AL: "IDENTIFICATION OF RAPD MARKERS LINKED TO A FERTILITY RESTORER GENE FOR THE OGURA RADISH CYTOPLASMIC MALE STERILITY OF RAPESEED (BRASSICA NAPUS L.)" THEORETICAL AND APPLIED GENETICS, vol. 88, no. 6/07, 1994, pages 741-748, XP002043620 cited in the application see the whole document	1-30

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of theinternational search	Date of mailing of the international search report
21 September 1998	08/10/1998

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3

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INTERNATIONAL SEARCH REPORT

national Application No
PCT/CA 98/00511

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